Remarks

Reconsideration of this Application is respectfully requested.

Upon entry of the foregoing amendment, claims 1-3, 5-9, 11 and 13-45 are pending in the application, with 1 being the independent claim. Support for the amendment to claim 1 can be found in original claim 4. These changes are believed to introduce no new matter, and their entry is respectfully requested.

Based on the above amendment and the following remarks, Applicant respectfully requests that the Examiner reconsider all outstanding objections and rejections and that they be withdrawn.

Rejections under 35 U.S.C. § 112

35 U.S.C. § 112, first paragraph

The Examiner has rejected claims 1-45 under 35 U.S.C.. §112, first paragraph, the Examiner alleged that the prior amendments to the claims insert new matter that is not supported by the specification. (Office Action pages 2-4, hereinafter "OA.") Specifically, the Examiner alleges that the specification does not have support for a "composition comprising a compound selected from the group consisting of mixtures thereof." (OA at page 3.) Applicant respectfully traverses this rejection.

An objective standard for determining compliance with the written description requirement of 35 U.S.C. §112, first paragraph, is, "does the description clearly allow persons of ordinary skill in the art to recognize that he or she invented what is claimed?" *In re Gosteli*, 872 F.2d 1008, 1012, 10 USPQ2d 1614, 1618 (Fed. Cir. 1989), *see also* MPEP 2163.02 (2004). An important consideration in assessing written description of a

claimed invention is the knowledge of one skilled in the art. *See Bilstad v. Wakalopulos*, 386 F.3d 1116, 1126 (Fed. Cir. 2004). The disclosure of a species has also been found to be sufficient to support a claimed genus when the disclosure of species would lead a person of ordinary skill to the genus. *See In re Herschler*, 591 F.2d 693 (CCPA 1979); *see also* MPEP 2163.05 (2004). In *Herschler,* the Court held that the disclosure of one corticosteroid was sufficient to support "physiologically active steroid" because the use would lead one of ordinary skill to the entire class of compounds. 591 F.2d at 697.

Applicant respectfully disagrees with the Examiner's position. In this case, the specification provides adequate descriptive support for "mixtures thereof" recited in presently-pending claim 1. Specifically, paragraph [0008] of the published application provides support for "mixtures thereof." The specification provides that the present invention encompasses "mixing a block copolymer with a population of polynucleotide molecules, a cationic surfactant, and an amorphous cryoprotectant or a bulking agent or any combination thereof, at a temperature below the cloud point of the block copolymer to form a mixture." (*See* paragraph [0008]) (emphasis added.) Furthermore, paragraphs [0082] and [0084] provide lists of amorphous cryoprotectants and crystalline bulking agents. Because paragraph [0008] indicates that these components can be used in "any combination thereof," there is support for "mixtures thereof." Applicants respectfully request that the Examiner reconsider and withdraw the rejection.

35 U.S.C. § 112, second paragraph

The Examiner has rejected claim 13 under 35 U.S.C. §112, second paragraph, the Examiner asserts that claim 13 lacks antecedent basis for the limitation "said amorphous cryoprotectant or crystalline bulking agent." (OA at page 4.) In response, Applicant has

amended claim 13 to delete the phrase "said amorphous cryoprotectant or crystalline bulking agent." Accordingly, Applicant respectfully requests that the Examiner reconsider and withdraw the rejection of claim 13.

Rejections under 35 U.S.C. § 103

Claims 1, 2, 5, 8-13, 15-24, 27-32, 37-39 and 40-45

The Examiner has rejected claims 1, 2, 5, 8-13, 15-24, 27-32, 37-39 and 40-45 under 35 U.S.C. § 103(a) as allegedly being unpatentable over Evans (WO 02/00844) in view of Volkin *et al.* (WO97/40839; hereinafter "Volkin"). The Examiner asserts that it would have been *prima facie* obvious to apply the disaccharide compounds and the crystalline bulking agents of Volkin to the methods of preparing a lyophilized composition of Evans to arrive at the presently claimed methods. (OA at page 8.) Applicant respectfully traverses this rejection.

The factors to be considered under 35 U.S.C. § 103(a), are the scope and content of the prior art; the differences between the prior art and the claims at issue; and the level of ordinary skill in the pertinent art. *See Graham v. John Deere*, 86 S.Ct. 684 (1966) and MPEP §2141. This analysis has been the standard for 40 years, and remains the law today. *See KSR International Co v. Teleflex Inc.*, 127 S.Ct. 1727 (2007).

A *prima facie* case of obviousness may be rebutted by a showing that the references or art at the time the invention was made teaches away from making a particular combination. "The court relied on the corollary principle that when the prior art teaches away from combining certain known elements, discovery of a successful means of combining them is more likely to be nonobvious." *See KSR International Co v.*

Teleflex Inc. , 127 S.Ct. 1727, at 1740 (2007), citing *United States v. Adams* 383 U.S. 39, at 51-52 (1966).

Additionally, objective evidence or secondary considerations such as unexpected results, commercial success, long-felt need, failure of others, copying by others, licensing, and skepticism of experts are relevant to the issue of obviousness and must be considered in every case in which they are present. *See* MPEP §2141. Here, even if the references could be properly combined under *Graham* (which they cannot) and disclosed all of the elements of the presently claimed invention (which they do not), the present invention provides at least one secondary indicia of nonobviousness over such a theoretical combined disclosure of the cited art - unexpected results. This has long been recognized as classical secondary indicia of nonobviousness, and is evident in the present case. *See Graham v. John Deere Co.*, 86 S.Ct. 684, 694 (1966); *Custom Accessories v. Jeffrey-Allan Industries*, 807 F.2d 955, 960 (Fed. Cir. 1986); *In re Soni*, 54 F.3d 746, 750 (Fed. Cir. 1995).

Summary of the references

Evans does not teach a method of producing a cationic surfactant, block copolymer and polynucleotide formulation and cold filtering the mixture followed by lyophilizing the mixture. All formulations described by Evans were prepared by mixing the pure copolymer with cold plasmid DNA. BAK is then added to the cold DNA/copolymer mix. In addition, Evans does not teach cold filtering of the mixture to produce a sterile formulation. Evans filters the components separately before combining them into a mixture.

Volkin teaches that DNA vaccine formulation containing sucrose and lactose greatly stabilize the DNA during lyophilized storage. Stability is measured by measuring the percentage of DNA that maintains the supercoiled structure as compared to the open circular or linear DNA structures, both structures are indicative of degraded DNA. Furthermore, the reference clearly indicates that while sucrose and lactose can stabilize DNA "mannitol does not enhance DNA stability compared to solution control (in PBS)." (Volkin page 81, lines 11-14.) Volkin does not teach that the addition of an amorphous cryoprotectant helps stabilize the particle size and maintains population polydispersity that remains unchanged during the freeze-drying process. Additionally, Volkin does not teach the use of cationic surfactant in combination with a block copolymer to stabilize the DNA formulation.

Not all elements are taught in the cited references

The presently claimed methods are directed to mixing a cationic surfactant, a polyoxyethylene (POE) and polyoxypropylene (POP) block copolymer, a polynucleotide and a compound selected from the group consisting of monosaccharides, disaccharides, oligosaccharides, sorbitol, hydrophilic polymers, proteins and mixtures thereof, at a temperature below the cloud point of said block copolymer to form a mixture, followed by cold filtering the mixture to produce a sterile formulation. Example 1 of the present application describes the production of sterile formulations using the method of claim 1.

The cited references do not disclose all elements of the presently claimed invention that is directed to a process of cold filtering a mixture comprising a cationic surfactant, copolymer, polynucleotide, and a compound selected from the group consisting of monosaccharides, disaccharides, oligosaccharides, sorbitol, hydrophilic

polymers, proteins and mixtures thereof. The missing element in the cited references is filtering the combined mixture using a single filter. The cited references teach filtering the individual components before combining them into a mixture. Here, Applicant discovered that the mixture of a cationic surfactant, copolymer, polynucleotide, and a compound selected from the group consisting of monosaccharides, disaccharides, oligosaccharides, sorbitol, hydrophilic polymers, proteins and mixtures thereof, disaccharides, oligosaccharides, sorbitol, hydrophilic polymers, proteins and/or mixtures thereof, can be filtered followed by lyophilization and then reconstituting the mixture without detriment to the particle formation. Thus, the references fail to teach all the elements of the presently claimed invention.

Teaches away

The cited references teach away from sterile filtering a combination of cationic surfactant, DNA and copolymer mixture. Evans teaches that the combination of BAK and DNA forms complexes or precipitates that are too large to filter. (*See* Evans page 2, lines 27-28; citing Musunuri *et al.* WO 99/21591, cited by the Examiner in OA at page 14.) Specifically, the incorporated reference WO 99/21591 teaches complexing BAK and DNA for the purpose of formulating a composition that can be used to introduce DNA into a host or host cell. In order to prepare a sterile formulation, that may be administered to an animal, the DNA and BAK stock solutions are filtered separately before the DNA and BAK are combined. (See WO 99/21591, Example 4.) WO 99/21591 teaches that once DNA and BAK is mixed it will either form a vesicular complex or a precipitate in aqueous solution. BAK alone does not form a vesicular structure or precipitate in aqueous solution and neither does DNA. Depending on the

concentration of BAK in the BAK-DNA mixture, either a vesicular complex ranging in size from 50-400 nm will form or the mixture will form a snowy flocculent precipitate. (*See* WO 99/21591, Example 2.) Thus, at the time the invention was made the ordinary artisan would have expected that a combination of cationic surfactant and DNA will result in the formation of vesicles and/or precipitates that cannot be filtered when combined together, and that the DNA and cationic surfactant would have to be filtered separately.

Obviousness cannot be predicated on what is not known at the time an invention is made, even if the inherency of a certain feature is later established. *In re Rijckaert*, 9 F.2d 1531 (Fed. Cir. 1993). Even after the filing date, Evans (EXHIBIT A) the same author of the cited WO 02/00844 reference asserted that:

> "Plasmid DNA and BAK surfactants form precipitates that have been reported to enhance DNA delivery. Based on this report, an unexpected result from our studies is that DNA-BAK precipitates do not coexist with CRL-1005-BAK-DNA particles (ternary complexes) in these formulations above the cloud point of CRL1005. However, DNA-BAK precipitates were observed in the D118 formulation below the cloud point (in the absence of CRL particles)." (*See* EXHIBIT A, page 1937, column 1, 2nd paragraph.)

The post filing date reference establishes that Evans at the time of filing the International Published Application No. WO 02/00844 did not appreciate that the mixture of DNA-BAK-CRL1005 does not form precipitates when mixed together above the cloud point. Evans does not make a sterile solution by filtering the combined mixture below the cloud point. "Preparation of sterile vaccine formulations requires only the addition of sterile BAK to a solution of DNA/CRL1005 that is sterile filtered below the cloud point." (*See* EXHIBIT A, page 1937, column 1, 3rd paragraph.) Thus, Evans filters two components the DNA-CRL1005 mixture and the BAK solution separately

before combing them to from a mixture. This is differs from the claimed invention which mixes all components and then uses only a single cold filtration step.

Unexpected results

Applicants have discovered that the process of making a sterile polynucleotide solution can be simplified by combining BAK-DNA-copolymer below the cloud point of the copolymer and sterile filtering the mixture, packaging the mixture and storing the mixture. (*See* published application [0066].) Applicants have unexpectedly discovered that microparticle formation does not need to occur prior to sterilization and storage. (*See* published application [0066].) Contrary to the Examiner's assertion the ordinary artisan would not have been motivated to combine the teachings of Evans and Volkin to arrive at the instantly claimed method of producing a sterile formulation of cationic surfactant, polynucleotide and copolymer. The ordinary artisan at the time the invention was made did not appreciate that a combination of DNA-BAK in the presence of a copolymer and an amorphous cryoprotectant does not form the expected BAK-DNA precipitate. As such, Applicant respectfully asserts that a *prima facie* case of obviousness has not been established and respectfully request that the Examiner reconsider and withdraw the rejection.

Claim 3

The Examiner has rejected claim 3 under 35 U.S.C. § 103(a) as allegedly being unpatentable over Evans (WO 02/00844) and Volkin (WO 97/40839) in view of Balasubramanian (U.S. Pat. No. 5,824,322.) The Examiner asserts that Balasubramanian teaches using a reverse triblock copolymer. (OA at page 10.) The teachings of Evans and Volkin are described above. Balasubramanian teaches "formulations that are

presented and stored in freeze-dried (lyophilized) conditions only requiring the addition of sterile water prior to use." (OA at page 10.) Applicant respectfully traverses this rejection.

Balasubramanian does not rectify the deficiencies of Evans and Volkin. In order to properly combine references there must be "some articulated reasoning with some rational underpinning to support a legal conclusion of obviousness." *See KSR International Co v. Teleflex Inc.* , 127 S.Ct. 1727, at 1741 (2007), citing *In re Kahn,* 441 F.3D, 977, 988 (C.A.Fed. 2006.) Balasubramanian teaches the use of reverse tri-block copolymers with various immunogens. (*See* columns 17 and 18.) However, Balasubramanian is silent with regards to formulating the reverse tri-block copolymer with a polynucleotide, let alone in combination with a cationic surfactant. In addition, Balasubramanian is silent with regards to cold filtering a reverse tri-block copolymer composition. Additionally, there are no suggestion in Balasubramanian to sterile filter the mixture before lyophilizing the mixture. Applicant asserts that the Examiner has failed to establish a *prima facie* case of obviousness in combining the references. As such, Applicants respectfully requested reconsideration and withdrawal of the rejection.

Claims 4, 6-7 and 25-26

The Examiner has rejected claims 4, 6-7 and 25-26 under 35 U.S.C. § 103(a) as allegedly being unpatentable over Evans (WO 02/00844) and Volkin (WO 97/40839) in view of Hunter *et al.* (U.S. Pat. No. 5,811,088, hereinafter "Hunter.") The Examiner asserts that that the combination of references teaches a "composition comprising POE and POP block copolymer; a polynucleotide; a cationic surfactant; and an amorphous cryoprotectant or a crystalline bulking agent produced with a cold filtration step as

claimed." (OA paragraph spanning pages 12-13.) Applicant respectfully traverses this rejection.

The teachings of Evans and Volkin are described above. Hunter teaches sterile filtering a copolymer, using a 0.22 μm filter, below the cloud point of the copolymer. Hunter does not teach a mixture comprising a copolymer and polynucleotide, or a copolymer and BAK, or a copolymer and a compound selected from the group consisting of monosaccharides, disaccharides, oligosaccharides, sorbitol, hydrophilic polymers, proteins and mixtures thereof. Thus, Hunter does not teach a composition comprising a mixture as presently claimed.

The deficiencies of Evans and Volkin are not cured by the disclosure of Hunter. Hunter does not disclose, suggest or otherwise contemplate a method of producing a sterile cationic surfactant, block copolymer and polynucleotide formulation. As such, taken together Evans, Volkin and Hunter do not teach all elements of the claimed invention. The invention is drawn to mixing a polynucleotide, a cationic surfactant, a copolymer, and a compound selected from the group consisting of monosaccharides, disaccharides, oligosaccharides, sorbitol, hydrophilic polymers, proteins and mixtures thereof, at a temperature below the cloud point of the copolymer and filtering the combined mixture. Applicant respectfully asserts that this combination of references is insufficient to establish a *prima facie* case of obviousness and respectfully request that the Examiner reconsider and withdraw the rejection.

Unexpected results

Applicants have unexpectedly discovered that microparticle formation does not need to occur prior to sterilization and storage. Applicants discovered that the mixture of

DNA, BAK and copolymer can be sterile filtered below the cloud point of the solution, and that the mixture can be aliquoted into sterile vials before frozen storage. (*See* published application [0066].) Thus, Applicants discovery that the combination of polynucleotide, cationic surfactant and copolymer mixture can be filtered is unexpected because it was not known that a DNA and cationic surfactant mixture does not form a precipitate in this combination. Additionally, it was not known beforehand that including the amorphous cryoprotectant with the DNA prevents a shift in particle size and helps maintain the same particle size before and after lyophilization. (*See* published application [0066].) Applicant respectfully requests that the Examiner reconsider and withdraw the rejection.

Claims 11-14

The Examiner has rejected claims 11-14 under 35 U.S.C. § 103(a) as allegedly being unpatentable over Evans (WO 02/00844) and Volkin (WO 97/40839) in view of Musunuri *et al.* (WO 99/21591, hereinafter "Musunuri.") The Examiner asserts that Musunuri teaches polynucleotide mixtures comprising sucrose at a concentration of 0 to about 9.25% w/v. (OA at page 14.) It would therefore, be *prima facie* obvious to include sucrose at about 10% w/v. (OA at page 15.) Furthermore, the Examiner asserts "that different concentrations of sucrose are viewed as merely optimizing the experimental parameters and not impacting patentability." (OA at page 16.) Applicant respectfully traverses this rejection.

The teachings of Evans and Volkin are described above. Musunuri teaches methods of delivering polynucleotide sequences to cells. Musunuri teaches using a mixture comprising polynucleotides, a cationic surfactant and sucrose.

Musunuri does not rectify the deficiencies of Evans and Volkin. Musunuri does not teach mixing a polynucleotide, a benzlammonium-containing surfactant and sucrose mixture with a co-polymer followed by cold filtering the mixture before lyophilizing the composition. Munsanai teaches that DNA and BAK mixtures will form snowy flocculant precipitates at BAK concentration above 0.04% w/v BAK. (*See* page 33, lines 5-8.) This precipitate is undesirable and does not induce as strong of a humoral immune response. (*See* page 38, lines 17-28; and Figure 1.) Additionally, the reference teaches that the combination of BAK and DNA forms vesicular complexes similar to classical liposomes and cationic liposomes that can achieve particle sizes ranging from 50 nm to 230 nm in size. (*See* page 32, lines 16-26.)

Contrary to the Examiner's assertion, Example 1 does not show filtering a mixture comprising DNA and BAK. (OA at page 15.) "Before admixture, both solutions are preferably filtered conventionally for example, using a 0.22 μm Millex GV syringe filter." (*See* sentence spanning page 30-31.) The ordinary artisan reading Musunuri would not be motivated to filter a composition comprising BAK and DNA, because the mixture will form complexes having a diameter of 230 nm (0.23 μm), these complexes are large enough to clog the conventional syringe filter assembly. Thus, the ordinary artisan reading Musunuri would not consider that filtering a mixture of DNA and BAK would a viable option to ensure sterility of the composition, because the reference is clear that it filters the components separately. Applicants respectfully assert that this combination of references is insufficient to establish a *prima facie* case of obviousness. Applicant respectfully requests that the Examiner reconsider and withdraw the rejection.

Claims 33-36

The Examiner has rejected claims 33-36 under 35 U.S.C. § 103(a) as allegedly being unpatentable over Evans (WO 02/00844) and Volkin (WO 97/40839) in view of Felgner *et al.* (U.S. Pat. No. 5,459,127, hereinafter "Felgner") The Examiner asserts that Felgner teaches Bn-DHxRIE, DHxRIE-OAc) and Pr-DOctRIE-OAC. "[T]hese cationic lipids all have the same general structure. These cationic lipids are suitable for intracellular delivery of polynucleotides. . . . The lipid formulations are amenable to freeze-dry technique." (OA at page 17.) The Examiner further asserts that it would have been *prima facie* obvious include cationic surfactant in the method of preparing a lyophilized composition. (OA at page 17.) Applicant respectfully traverses this rejection.

The teachings of Evans and Volkin are described above. Felgner teaches DNA transfection protocols using cationic lipid-DNA complexes. Specifically, Felgner teaches using the following cationic surfactants Bn-DHxRIE, DHxRIE-OAc, DHxRIE-OBz or Pr-DOctRIE-OAc in the transfection methods.

Felgner does not rectify the deficiencies of Evans and Volkin. Felgner does not teach lyophilizing compositions comprising the cationic surfactants Bn-DHxRIE, DHxRIE-OAc, DHxRIE-OBz or Pr-DOctRIE-OAc. Felgner does not teach adding sucrose to the cationic surfactant DNA mixture. Felgner also does not teach adding a copolymer into the DNA cationic surfactant mixture.

Here, Applicants have unexpectedly discovered that microparticle formation does not need to occur prior to sterilization and storage. (*See* published application [0066].) Thus, Applicants discovery that the combination of polynucleotide, cationic surfactant

and copolymer mixture can be filtered is unexpected because it was not known that a DNA, cationic surfactant, and amorphous cryoprotectant mixture does not form a precipitate in this combination. Additionally, it was not known beforehand that including the amorphous cryoprotectant with the DNA prevents a shift in particle size and help maintain the same particle size before and after lyophilization. (*See* published application [0066].) Applicants respectfully assert that this combination of references is insufficient to establish a *prima facie* case of obviousness. Applicant respectfully requests that the Examiner reconsider and withdraw the rejection.

Conclusion

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicant therefore respectfully requests that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. Applicant believes that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

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Characterization and Biological Evaluation of a Microparticle Adjuvant Formulation for Plasmid DNA Vaccines

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Received 31 December 2003; revised 3 March 2004; accepted 17 March 2004

Published online 13 May 2004 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jps.20112

ABSTRACT: We describe the physiochemical characterization and immunological evaluation of plasmid DNA vaccine formulations containing a nonionic triblock copolymer adjuvant (CRL1005) in the presence and absence of a cationic surfactant, benzalkonium chloride (BAK). CRL1005 forms particles of 1–10 microns upon warming above its phase-transition temperature (~6–8°C) and the physical properties of the particles are altered by BAK. DNA/CRL1005 vaccines formulated with and without BAK were evaluated in rhesus macaques to determine the effect of CRL1005 and BAK on the ability of plasmid DNA to induce a cellular immune response. Immunogenicity results indicate that the addition of CRL1005 to human immunodeficiency virus-1 *gag* plasmid DNA formulated in phosphate-buffered saline leads to an enhancement in the gag-specific cellular immune response. Moreover, the addition of BAK to human immunodeficiency virus-1 *gag* plasmid DNA/CRL1005 formulations produces an additional enhancement in gag-specific cellular immunity. *In vitro* characterization studies of DNA/CRL1005 formulations indicate no detectable binding of DNA to CRL1005 particles in the absence of BAK, suggesting that the enhancement of cellular immunity induced by DNA/CRL1005 formulations is not due to enhanced DNA delivery. In the presence of BAK, however, results indicate that BAK binds to CRL1005 particles, producing cationic microparticles that bind DNA through electrostatic interactions. If BAK is present at the phase-transition temperature, it reduces the particle size from ~2 microns to ~300 nm, presumably by binding to hydrophobic surfaces during particle formation. Zeta potential measurements indicate that the surface charge of CRL1005-BAK particles changes from positive to negative upon DNA binding, and DNA bound to the surface of CRL1005-BAK particles was visualized by fluorescence microscopy. These results indicate that the addition of BAK to DNA/CRL1005 formulations leads to the formation of ~300 nm CRL1005-BAK-DNA particles that enhance the cellular immune response in rhesus monkeys. J Pharm Sci 93:1924–1939, 2004

Keywords: DNA; DNA delivery; vaccine adjuvants; microparticles; particle sizing; light-scattering; immunology

INTRODUCTION

Plasmid DNA-based vaccines have the potential for significant advances beyond both live virus and protein-based vaccines in terms of stability,

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Journal of Pharmaceutical Sciences, Vol. 93, 1924–1939 (2004)

safety and characterizability. However, large doses of DNA are required to induce sufficiently robust immune responses in humans and nonhuman primates.[1–4] Therefore, a need exists for the identification of safe and effective adjuvants to enhance the ability of plasmid DNA-based vaccines to stimulate both humoral and cellular immunity.

CRL1005 is a nonionic triblock copolymer composed of blocks of polyoxypropylene (POP) and polyoxyethylene (POE).[5] Linear triblock POE-POP-POE copolymers are referred to by the BASF trade name Pluronic® or by the term *poloxamer* and were originally developed by Wyandotte Chemicals Corporation and BASF Performance in the early 1950s, for use as surfactants.[5] The early copolymers had POP cores up to ~4,000 Da (from 16 to 67 POP blocks and from 2 to 122 POE blocks). The CRL series copolymers differ from the older Pluronic® copolymers by having a much larger molecular weight POP core (9–20 kDa POP core containing 155–345 POP blocks, and with 2.5–20% POE). CRL1005 has a POE content of 5% and a POP core of 12,000 Da. The larger molecular weight of the CRL copolymers endows them with unusual solubility properties. Typically, these copolymers are soluble in aqueous solutions at near ice temperatures but they aggregate into microparticles above their phase-transition (cloud-point) temperature. It is believed that the adjuvant properties of the CRL copolymers are related to their ability to aggregate into surface-active particles.[6,7]

Plasmid DNA vaccines formulated with CRL1005 have been shown to significantly enhance the levels of antigen-specific cellular immune responses in rhesus monkeys.[8] Moreover, the results indicate that a DNA/CRL1005 formulation containing benzalkonium chloride (BAK) induced even higher levels of $CD8^+$ gag-specific T cells than the DNA/CRL1005 formulation without BAK, during the priming series of three vaccinations.[9] These results suggest that the combination of CRL1005 and BAK is an effective adjuvant for plasmid DNA vaccines in rhesus monkeys.

We have characterized DNA/CRL1005 formulations in the presence and absence of BAK to determine how the physical properties of the formulation are altered by the presence of this cationic surfactant. We believe that understanding the effects of BAK on the DNA/CRL1005 formulation might suggest possible mechanisms of immunogenicity enhancement and guide the direction of future formulation development. Our results indicate that BAK binds to CRL1005 particles through hydrophobic interactions, producing cationic microparticles that subsequently bind DNA through electrostatic interactions.

In this report, we describe the effects of BAK on the size and surface charge of CRL1005 particles and show that DNA-BAK precipitates do not exist in these DNA/CRL1005/BAK formulations above the cloud point of CRL1005. We also demonstrate that other cationic surfactants may be used in place of BAK to enhance the binding of plasmid DNA to CRL1005 particles. These results show that CRL1005/cationic surfactant formulation composition and preparation methods can be easily manipulated to alter the particle size, charge, and amount of DNA bound to the surface, suggesting that this system may be useful to explore the effects of many different variables on the adjuvant properties of CRL1005.

EXPERIMENTAL

Materials

CRL1005 (TranzFect) was obtained from CytRx Corporation (Atlanta, GA). BAK (a mixture of several homologs of differing chain length) was purchased from Spectrum (New Brunswick, NJ) or from Ruger Chemical Co. (Irvington, NJ). Dodecyl trimethylammonium bromide (BAK-12), tetradecyl trimethylammonium bromide (BAK-14), and hexadecyl trimethylammonium bromide (BAK-16) were purchased from Sigma Chemical Co. (St. Louis, MO) for use as high-performance liquid chromatography (HPLC) reference standards. Cetyl pyridinium chloride (CPC) was obtained from Zeeland Chemical (Zeeland, Michigan) or from Spectrum. Cetyl trimethylammonium chloride (CTAC) was obtained from Spectrum and PicoGreen was purchased from Molecular Probes (Eugene, OR).

Vaccine Vector

A synthetic gene for *gag* from a Clade B sequence human immunodeficiency virus (HIV)-1 was previously constructed using codons frequently used in humans.[10,11] The *gag* gene was inserted into the V1Jns plasmid (V1Jns-gag) under the control of the human cytomegalovirus/human intron A promoter and bovine growth hormone terminator.[12,13]

Description of Formulations

The formulations used in the rhesus immunogenicity study and for most of the *in vitro* characterization studies were (1) D101; 5 mg/mL V1Jns-gag plasmid DNA in phosphate-buffered saline (PBS), (2) D113; 5 mg/mL V1Jns-gag plasmid DNA in PBS containing 7.5 mg/mL CRL1005, and (3) D118; 5 mg/mL V1Jns-gag plasmid DNA in PBS containing 7.5 mg/mL CRL1005 and 0.5–0.6 mM BAK.

Preparation, Handling, and Storage of Formulations Containing CRL1005

Plasmid DNA formulations containing CRL1005 were prepared by adding CRL1005 to an aqueous solution of plasmid DNA in PBS at room temperature, followed by the addition of BAK. Care was taken to ensure complete solubilization of the CRL1005 by performing several cycles of cooling on ice, vigorous vortexing, then warming above the phase-transition temperature with additional vortexing. Care was also taken to ensure complete solubilization of the CRL1005, by incubation on ice, just before −70°C storage. Before use for either immunization studies or the *in vitro* characterization studies, CRL1005-containing formulations were removed from −70°C storage then warmed above the cloud point by incubation at room temperature. Rapid thawing of the frozen CRL1005 formulations in a water bath was avoided because this causes a significant reduction in the particle size, particularly in formulations lacking BAK.

Cloud-Point Determinations

The cloud-point temperature of the D118 formulation was determined using a model 62DS Aviv Circular Dichroism Spectrometer. A temperature scan from −1 to 14°C was performed while recording the output CD-Dynode signal at a wavelength of 360 nm, to monitor the light-scattering intensity. The onset of particle formation (i.e., the temperature at which the CD-Dynode signal begins to increase above the baseline) was defined as the cloud point of the formulation. The cloud point of the D118 formulation by this method (6°C) is specific for this particular formulation and therefore does not correspond to the lower critical solution temperature of the CRL1005 polymer.

Immunization

Rhesus macaques were between 3–10 kg in weight. In all cases, the total vaccine dose was suspended in 1 mL of PBS. The macaques were anesthetized (ketamine/xylazine) and the vaccines were delivered intramuscularly (im) in 0.5-mL aliquots into both deltoid muscles using tuberculin syringes (Becton-Dickinson, Franklin Lakes, NJ). Sera and peripheral blood mononuclear cells (PBMCs) were prepared from blood samples collected at several time points during the immunization regimen. All animal care and treatment were in accordance with standards approved by the Institutional Animal Care and Use Committee according to the principles set forth in the Guide for Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council.

IFN-γ ELISPOT Assay

Ninety-six-well flat-bottomed plates (Immobilon-P membrane; Millipore) were coated with 1 μg/well of anti-gamma interferon (IFN-γ) MAb MD-1 (U-Cytech-BV) overnight at 4°C. The plates were then washed three times with R10 medium [RPMI (Gibco-BRL), 10% fetal bovine serum (HyClone), 50 μM 2-mercaptoethanol (Gibco-BRL), 1 mM HEPES (Gibco-BRL), 200 μM L-glutamine, 100 μM sodium pyruvate, penicillin-streptomycin (Gibco-BRL)] and then the plates were blocked in R10 for 2 h at 37°C. The solution was discarded from the plates, and freshly isolated PBMCs were added at $2–4 \times 10^5$ cells/well. The cells were stimulated in the absence (mock) or presence of the HIV-1 *gag* peptide pool (4 μg/mL per peptide) or concanavalin A (Sigma) per milliliter. Cells were then incubated for 20–24 h at 37°C in 5% CO_2. Plates were washed six times with PBST [PBS (Gibco-BRL) containing 0.05% Tween 20 (Sigma)], and 1 μg of rabbit anti-IFN-γ polyclonal biotinylated detector antibody solution (U-Cytech-BV) per well was added, and the plates were incubated overnight at 4°C. The plates were washed six times with PBST and 1:2500 dilution of streptavidin-alkaline phosphatase conjugate (Pharmingen) was added per well. Color was developed by incubating in NBT/BCP (Nitro-blue tetrazolium chloride/5-Bromo-4-chloro-3′-indolyphosphate p-toluidine salt) (Pierce) for 10 min. Spots were counted under a dissecting microscope and normalized to 1×10^6 PBMCs.

Static Laser Diffraction Light Scattering

Particle-size distributions were generated by static laser diffraction light-scattering analysis of samples at room temperature using a Mastersizer 2000 (Malvern Instruments). Because of the high turbidity of the CRL1005 formulations, samples were diluted ~100-fold with degassed and 0.22-μm filtered PBS before the measurement. Degassed PBS was used for a background control measurement. Care was taken to use gentle mixing of diluted samples to avoid the generation of bubbles. A refractive index of 1.45 was used for the light-scattering measurements of CRL1005-containing formulations.

Zeta Potential and Dynamic Light Scattering

Zeta potential measurements and particle-size analyses by dynamic light scattering, shown in Table 1, were determined using a Zetasizer 3000 (Malvern Instruments). All samples were diluted 46-fold with 20 mM Tris-acetate (pH 7.2) before zeta potential measurements. The particle-size measurements of samples containing CRL1005 were performed after diluting samples by 50-fold with PBS. The particle size of plasmid DNA was determined at two concentrations, 0.2 and 2.5 mg/mL plasmid DNA in PBS. Data were collected for five 20-s cycles for each sample then averaged to yield the final value. As a control, a -50 ± 5 mV zeta potential standard (Malvern Instruments) was used.

Table 1. Surface Charge (Zeta Potential) and Hydrodynamic Diameter (Dh) of CRL1005 Formulations

Formulation[a]	Zeta Potential (mV)	Dh (nm)
DNA	−48.5	66, 62[b]
CRL1005	0.9	2178
CRL1005 + DNA	2.7	2468
CRL1005 + BAK	10.2	225
CRL1005 + BAK + DNA	−46.7	330

[a]The concentrations of DNA, CRL1005, and BAK were 5 mg/mL, 7.5 mg/mL, and 0.6 mM, respectively. Measurements were taken on CRL1005 samples after vigorous mixing below the cloud point to dissolve CRL1005, followed by warming above the cloud point to allow for particle formation. Samples were diluted before analysis as described in the Experimental section.

[b]Particle size of 0.2 and 2.5 mg/mL DNA, respectively, in PBS.

Adsorption of BAK to CRL1005 Particles

To determine the adsorption of BAK to CRL1005 particles, BAK at various concentrations was mixed either with cold (below cloud point) CRL1005 solution (7.5 mg/mL in PBS) or with a CRL1005 suspension at room temperature. After a 1-h incubation at room temperature, 1 mL of the mixture was centrifuged at 25°C for 1 h at 440,000*g*. The BAK concentration in the supernatant was determined using UV absorption and second-derivative analysis in the range of 230–300 nm using methodology developed previously for the analysis of protein and DNA mixtures.[14] A Hewlett-Packard 8453 diode-array spectrophotometer controlled by a personal computer equipped with the HP spectral analysis software was used. Solutions of BAK-12 and plasmid DNA in PBS were used as the reference standards for the analysis. The amount of adsorbed BAK was obtained by subtracting the BAK concentration in the supernatant from the total BAK concentration in the mixture.

BAK Composition Analysis

The homolog composition of BAK (Spectrum) was determined by reverse-phase HPLC (RP-HPLC) using an isocratic separation method on a YMC-Pack CN (cyano) 120A S-5 4.6 × 250 mm column at room temperature (Waters, Milford, MA). The mobile phase contained 55% acetonitrile and 45% 0.1 M sodium acetate at pH 5.0. BAK samples were injected at a flow rate of 1 mL/min and the UV absorbance was monitored at 260 nm. The chromatograms were transferred to an Excel spreadsheet for quantitative analysis. UV absorbance peaks were identified by comparison to BAK-12, BAK-14, and BAK-16 reference standards.

DNA Association to CRL1005-BAK Particles

The adsorption of DNA to CRL1005 particles was determined using a filtration method and/or by sucrose density gradient centrifugation. For the filtration method, 3- to 5-mL samples were filtered through 25-mm syringe filters with a pore size of 0.1 μm (MILLEX-VV; Millipore). The DNA concentration in the filtrate (free DNA concentration) was determined by the UV absorbance at 260 nm. The amount of DNA bound to CRL1005-BAK particles was calculated by subtracting the

free DNA from total DNA. Filtration of control samples containing only DNA in PBS indicated no detectable DNA adsorption to the filter.

A sucrose density gradient centrifugation method was also used to determine the association of DNA to CRL1005 particles. For BAK-containing samples, a 3.2-mL gradient containing 2–13% sucrose and 25 mM NaCl was used. Samples (0.2 mL) were layered on top of the gradient and centrifuged at 80,000 rpm (346,000*g*) for 1.5 h to pellet the free DNA. After centrifugation, 16 gradient fractions (0.2 mL) were collected and analyzed for DNA concentration by first dissolving the CRL1005 polymer with sodium dodecyl sulfate (to 1% w/w), then using a UV multicomponent analysis method to determine the DNA concentration.[14] Control studies conducted with samples containing CRL1005, DNA, DNA/CRL1005, and DNA/CRL1005/BAK in PBS indicated that the CRL1005 polymer remained in the upper 6–8 fractions of the gradient after centrifugation but that essentially all (>99%) of the free DNA was pelleted (data not shown). For samples containing CPC, CTAC, or high concentrations (>1.2 mM) of BAK, a 12–18% sucrose gradient in 25 mM NaCl was utilized. Higher levels of DNA association in these samples increased the density of the DNA-CRL1005-surfactant particles such that they would pellet through the 2–13% sucrose gradient. Centrifugation of the 12–18% gradient was performed at 346,000*g* for 4 h.

Microscopy

The morphology of the particles in CRL1005 formulations and DNA association to the particles were imaged using an Olympus IX71 microscope equipped with a Spot digital camera. Three microliters of the formulation was placed on a slide, covered with a glass coverslip, and sealed with nail polish. A polarizer condenser and a 100-power objective were used for bright field imaging of the particles to determine morphology. To visualize DNA association, large particles of CRL1005 (~2 μm) were formed by slowly warming a cold solution of 7.5 mg/mL CRL1005 in PBS above the cloud point. BAK, PicoGreen, and plasmid DNA were then added, in that order. The DNA concentration was lowered to 0.3 mg/mL to reduce the background fluorescence, and the BAK and PicoGreen concentrations were 0.6 mM and 1:1000 (v/v), respectively. The excitation/emission cube for fluorescein isothiocyanate was used for DNA/PicoGreen fluorescence.

Assay for DNA-BAK Precipitates in DNA/CRL1005/BAK Formulations

DNA-BAK precipitates were separated from free DNA by centrifugation as described below. One milliliter of each formulation was centrifuged (at 25°C) in a Beckman Ultracentrifuge with a fixed-angle rotor for 30 min at 5, 10, 15, 20, 25, and 35 thousand rpm (1.4 k, 5.4 k, 12.3 k, 21.8 k, 34 k, and 66.5 k*g*, respectively). DNA-BAK precipitates were completely pelleted at 20,000 rpm; however, free DNA did not start to pellet until the speed reached 35,000 rpm (~10% of the free DNA was pelleted at 35,000 rpm). There was no significant sedimentation of CRL1005-BAK-DNA particles at or below 35,000 rpm (data not shown). Pelleted precipitates of DNA-BAK were dissolved in 2% sodium dodecyl sulfate before UV spectroscopy to determine the DNA concentration.

RESULTS

Immunogenicity of CRL1005 Formulations in Rhesus Macaques

To evaluate the ability of CRL1005 and CRL1005-BAK to adjuvant the immune response induced by plasmid DNA, the immunogenicity of adjuvanted and unadjuvanted formulations of V1Jns-gag plasmid DNA in rhesus monkeys were compared. The results in Table 2 show that the addition of 7.5 mg/mL CRL1005 to the control formulation containing only 5 mg/mL V1Jns-gag DNA in PBS enhanced the gag-specific cellular immune response, based on an increase in IFN-γ-secreting T cells after the second immunization (at 6 weeks). However, the strongest immune responses were generated by a formulation containing 5 mg/mL DNA formulated with 7.5 mg/mL CRL1005 and 0.5 mM BAK. These results clearly show that BAK enhanced the immunogenicity of V1Jns-gag plasmid DNA.

Binding of BAK to CRL1005 Particles

To determine the effects of BAK on the size and morphology of CRL1005 particles, we formulated 5 mg/mL DNA with 7.5 mg/mL CRL1005 (formulation D113) and evaluated the effect of adding 0.6 mM BAK (to make formulation D118), using visual microscopy. As shown in Figure 1A, the CRL1005 particles in the absence of BAK were found to be quite uniform in size, with the

Table 2. Effect of CRL1005 and BAK on the Immune Response Induced by HIV *gag* Plasmid DNA in Rhesus Monkeys[a]

	Gag-Specific IFN-γ ELISPOT Response (SFC per 10^6 PBMCs) at Week:				
Vaccine Formulation	0	4	6	8	12
5 mg/mL *gag* DNA	0	5	33	28	139
	1	9	81	9	7
	3	10	82	26	96
	1	30	82	45	71
	1	**14**	**70**	**27**	**78**
5 mg/mL *gag* DNA	18	19	116	61	182
7.5 mg/mL CRL1005	4	39	168	125	283
	0	13	219	98	122
	4	7	125	57	216
	7	**20**	**157**	**85**	**201**
5 mg/mL *gag* DNA	0	108	530	366	380
7.5 mg/mL CRL1005	7	486	671	374	1405
0.5 mM BAK	2	5	111	31	53
	0	54	152	77	161
	2	**163**	**366**	**212**	**500**

[a]Plasmid DNA (5 mg) was injected im into four rhesus monkeys per group at 0, 4, and 8 weeks. At the indicated times, the gag-specific T cells were quantified using the IFN-γ ELISPOT assay as described in the Experimental section. Results shown are net responses after subtraction of spots formed in medium control wells. The mean response for each group is shown in bold typeface for each time point. SFC = spot forming cells.

majority of the particles being <5 microns in diameter. However, the addition of BAK reduced the apparent particle diameter to significantly below 2 microns. To confirm the effect of BAK on the particle size, we used static laser diffraction light scattering on the same plasmid DNA-containing formulations. The results (Fig. 1B) show that the mean CRL1005 particle size in the absence of BAK (D113) was ~2–3 microns, whereas in the formulation containing BAK (D118), the CRL1005 particles had a diameter of ~300 nm. We also prepared a formulation containing 5 mg/mL DNA and 0.6 mM BAK in PBS (without CRL1005) to determine the potential for generating DNA-BAK precipitates in D118. The results indicate that DNA-BAK particles were present (without CRL1005) and, based on the particle-size distribution shown in Figure 1B, most of volume of the particles was associated with particles having a diameter in the 100- to 1000-micron range. The cloudy visual appearance of this formulation also confirmed the presence of large particles.

To examine the binding of BAK to CRL1005 particles, BAK was mixed with 7.5 mg/mL CRL1005 in PBS either before or after particle formation and the amount of BAK bound to the CRL1005 particles was determined as described in Experimental. The results in Figure 2 show that ~50% of the input BAK was bound to CRL1005 particles and that the amount of BAK bound was the same regardless of whether the BAK was present during particle formation or was added after particle formation. The results also indicate that the percentage of BAK bound to CRL1005 particles was slightly higher in samples prepared with BAK from Ruger Chemical, compared with samples containing BAK from Spectrum, suggesting that the BAK composition affects binding to CRL1005 particles (discussed below).

Light-scattering measurements indicated that the hydrodynamic diameter of CRL1005 particles formed in the presence of BAK was ~300 nm (Fig. 1B). However, when CRL1005 particles were formed in the absence of BAK then mixed with BAK, the particle size before and after BAK addition was the same (2.6 microns). Therefore, the results in Figure 2 show that the binding of BAK to CRL1005 particles is independent of surface area, because the particle size (and surface area) did not affect the amount of BAK bound to the particles. The data suggest that BAK not only binds to the surface of the particles but also penetrates into (or is soluble in) the interior of the CRL1005 particle. Measurements of the partition coefficient of BAK, between a pure CRL1005 polymer phase and an

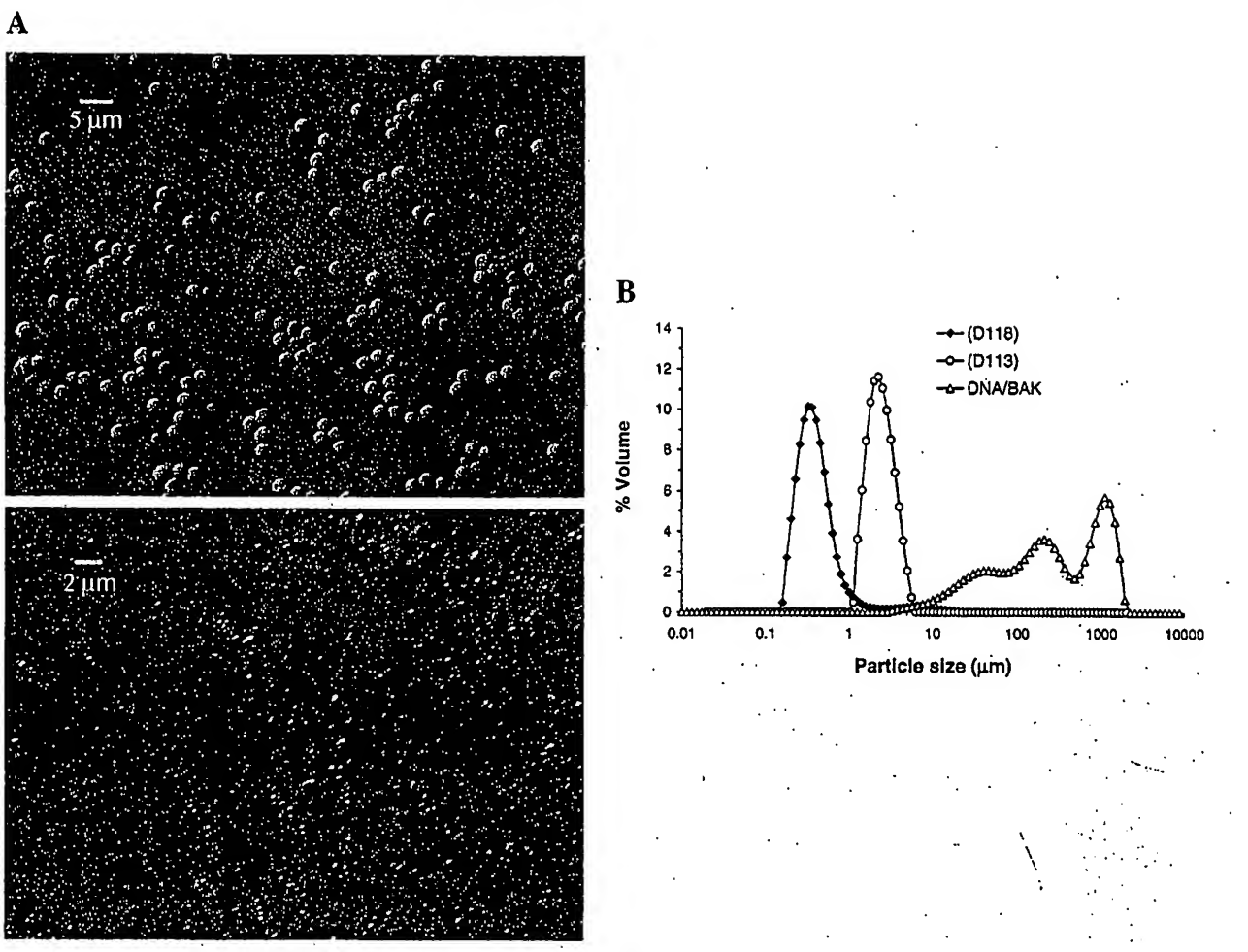

Figure 1. Morphology and size distribution of CRL1005 particles. (A) The morphology of CRL1005 particles was visualized by microscopy as described in the Experimental section. In a PBS formulation containing 5 mg/mL plasmid DNA and 7.5 mg/mL CRL1005 but no BAK (D113), the CRL1005 particles had a smooth and spherical morphology with a diameter of ~2 µm (upper panel). In a PBS formulation containing 5 mg/mL plasmid DNA, 7.5 mg/mL CRL1005 and 0.6 mM BAK (D118), the CRL1005 particle size was reduced to <0.5 µm (lower panel). (B) Particle-size distribution determined by static laser diffraction light scattering. The DNA/BAK mixture contained 5 mg/mL DNA and 0.6 mM BAK. Each formulation was warmed from −70°C storage to room temperature, then diluted with PBS before the particle-size measurement.

aqueous phase containing PBS, strongly suggest that BAK preferentially partitions into the CRL1005 polymer. The partition coefficients for BAK-12 and BAK-14 into the CRL1005 phase are 59 and 380, respectively.

Effect of BAK Chain Length on Binding to CRL1005 Particles

The results in Figure 2 indicate that BAK binds to CRL1005 particles. However, commercially available BAK is a mixture of several homologs with the hydrocarbon chain having lengths of 12, 14, 16, and sometimes 18 carbons. Because hydrophobic interactions are the likely driving force for the binding of BAK to CRL1005 particles, we determined the effect of BAK chain length on its ability to associate with particles of CRL1005. Our approach relied on the use of RP-HPLC to analyze the BAK in the unbound fraction, after centrifugation to pellet CRL1005 particles with bound BAK. The results shown in Table 3 indicate that >95% of the unbound BAK fraction was BAK-12, showing that the longer chain-length

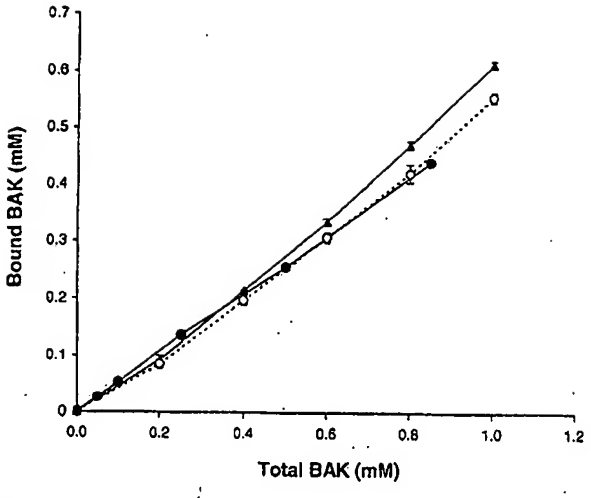

Figure 2. Adsorption of BAK to CRL1005 particles. In solutions containing CRL1005 and varying concentrations of BAK (without DNA), the CRL1005 particles with adsorbed BAK were pelleted by centrifugation, and the free BAK concentration in the supernatant was determined by UV absorption and second-derivative analysis, as described in the Experimental section. BAK at the designated concentrations and CRL1005 at 7.5 mg/mL were mixed using two different methods: (1) The CRL1005/BAK mixture was chilled on ice to obtain a clear solution, then warmed to room temperature, allowing CRL1005 particles to form in the presence of BAK (from Spectrum). The CRL1005 particles formed under these conditions had a diameter of ~300 nm (○); (2) CRL1005 in PBS at 7.5 mg/mL was chilled on ice to obtain a clear solution, then warmed to room temperature to allow the CRL1005 polymer to form ~2-μm particles in the absence of BAK. After particle formation, 0.6 mM BAK (from Spectrum) was added with gentle mixing (●); (3) same as (2) except BAK was from Ruger Chemical (▲).

homologs have a greater tendency to associate with CRL1005 particles. Comparing these results with those in Figure 2 indicates that the percentage of BAK bound to the CRL1005 particles was ~25%, ~90–95%, and ~100% for BAK-12, BAK-14, and BAK-16, respectively, in the presence and absence of 5 mg/mL plasmid DNA. These results strongly suggest that hydrophobic interactions are the major driving force for association of BAK to CRL1005 particles. The composition of BAK from Ruger Chemical (50% BAK-14-18, composition provided by the vendor) has a somewhat larger fraction of the longer chain-length homologs than Spectrum BAK (~33% BAK-12-16), as measured by RP-HPLC. Based on the results in Table 3 and the known compositions of Spectrum and Ruger BAK, we would predict that a larger fraction of the total BAK would be bound to CRL1005 particles for Ruger BAK. The results in Figure 2 are consistent with this prediction.

Table 3. Composition of Unbound BAK in CRL1005 Formulations

	Percent of each BAK Homolog		
Sample	BAK-12	BAK-14	BAK-16
Spectrum BAK control	67.4	24.2	8.3
Supernatant A[a]	97.9	1.9	0.2
Supernatant B[b]	96.7	2.7	0.7

[a]Supernatant from a sample containing 7.5 mg/mL CRL1005 and 0.85 mM Spectrum BAK.
[b]Supernatant from a sample containing 7.5 mg/mL CRL1005, 0.85 mM Spectrum BAK, and 5 mg/mL DNA.

Effect of BAK on the Size and Surface Charge of CRL1005 Particles

The effect of BAK on the zeta potential and size of CRL1005 particles is shown in Table 1. The results show that the CRL1005 particles had a hydrodynamic diameter of ~2 microns and a surface charge close to neutrality, as expected from the nonionic structure of the polymer. The addition of 5 mg/mL DNA to the CRL1005 preparation resulted in only a minor change in the zeta potential and particle size. Moreover, the results with the DNA control show that the plasmid DNA in the CRL1005 formulations did not dominate the zeta potential or the particle-size measurements. The zeta potential of a formulation containing 0.6 mM BAK and 7.5 mg/mL CRL1005 was ~+10 mV, a value consistent with the data in Figure 2 showing that BAK binds to CRL1005 particles. The presence of BAK during particle formation also caused a large reduction in particle size. The addition of plasmid DNA to the CRL1005/BAK formulation produced particles with a zeta potential of ~−47 mV and a diameter near 330 nm. The highly negative zeta potential suggests that DNA binds to CRL1005-BAK particles, giving them a negative surface charge.

The effect of BAK and CRL1005 concentration on the zeta potential of CRL1005 particles in the presence of 5 mg/mL DNA is shown in Figure 3. At 7.5 mg/mL CRL1005, the results show that CRL1005 particles became negatively charged at ≥0.4 mM BAK but that there was no detectable surface charge at or below 0.3 mM BAK. These results suggested that a threshold of positive surface charge is required for binding of DNA to CRL1005-BAK particles. To further explore the

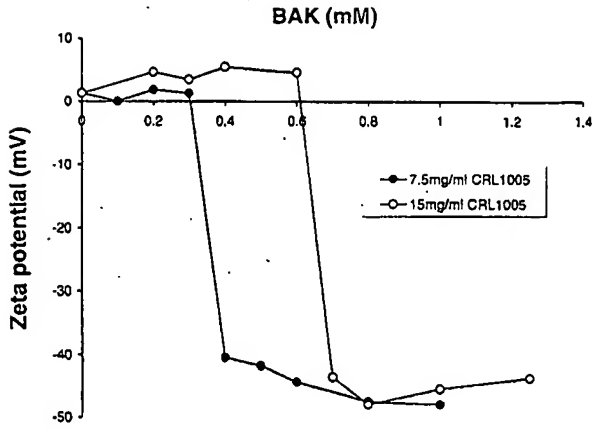

Figure 3. Effect of BAK and CRL1005 concentration on the zeta potential of CRL1005 particles. Formulations containing 7.5 or 15 mg/mL CRL1005, 5 mg/mL plasmid DNA in PBS, and varying concentrations of BAK were diluted and the zeta potential measurements made as described in the Experimental section.

nature of this threshold, we repeated the experiment using a twofold-higher concentration of CRL1005. We reasoned that if the threshold corresponds to the minimum positive surface charge required for DNA binding, then doubling the CRL1005 concentration would result in a doubling of the BAK concentration at the threshold. The results in Figure 3 are consistent with this hypothesis. However, the data in Figure 2 indicate that there was no threshold of BAK concentration required for BAK binding to CRL1005 particles. Therefore, the threshold observed in Figure 3 suggests that a minimum positive surface-charge density is required for the binding of DNA to CRL1005-BAK particles.

DNA Binding to CRL1005-BAK Particles

To further determine whether plasmid DNA binds to CRL1005 particles in the presence or absence of BAK, we subjected samples containing 7.5 mg/mL CRL1005, 5 mg/mL DNA, and various concentrations of BAK to sucrose gradient centrifugation analysis to separate unbound DNA from the CRL1005 polymer. UV spectroscopic analysis of fractions of the sucrose gradient after centrifugation revealed that there was no detectable DNA in the polymer containing fractions in the absence of BAK (Fig. 4A). However, there was a significant amount of DNA in the polymer containing fractions in the presence of BAK, suggesting that DNA binds to CRL1005 particles only in

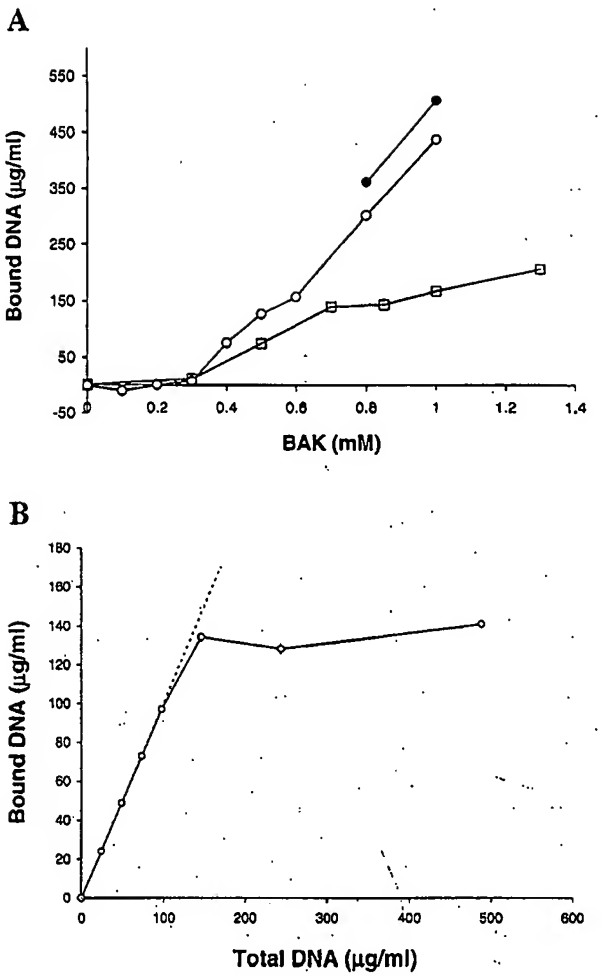

Figure 4. (A) Effect of BAK concentration on the amount of plasmid DNA associated with CRL1005 particles. Samples contained 7.5 mg/mL CRL1005, 5 mg/mL plasmid DNA, and the indicated concentrations of BAK. Free DNA was separated from DNA bound to CRL1005 particles using a sucrose density gradient centrifugation method (□) or a filtration method with 0.5 mg/mL DNA (○) and 5 mg/mL DNA (●). The amount of DNA bound to CRL1005 particles is expressed as micrograms/milliliter bound DNA with a total DNA concentration of 5 mg/mL. (B) Effect of DNA concentration on the amount of DNA bound to CRL1005 particles. DNA association to particles in a formulation containing 7.5 mg/mL CRL1005, 0.6 mM BAK, and the indicated concentration of DNA, was quantitated by filtration and UV spectroscopy, as described in the Experimental section. The dashed line represents expected value, assuming 100% binding of plasmid DNA.

the presence of BAK. The results also show that the amount of DNA associated with the CRL1005 particles increased with BAK concentration and that the amount of DNA bound at 0.5 mM BAK

(~75 μg/mL) represented about 1.5% of the total DNA. Because the amount of DNA bound to the polymer particles was such a small fraction of the total DNA, a second approach was used to confirm the presence of bound DNA, based on a filtration method to separate CRL1005 particles from free DNA. The results (Fig. 4A) show that DNA was retained by the filter only in the presence of BAK, suggesting that there was no significant binding of DNA to CRL1005 particles in the absence of BAK. However, the amount of DNA retained with the CRL1005-BAK particles was significantly higher using this technique than with the sucrose gradient centrifugation method (above 0.6 mM BAK). Because the background level of DNA retained by the filter in the absence of CRL1005 and BAK was affected by the DNA concentration, this titration was conducted at 0.5 mg/mL DNA. However, repeating the analysis at 0.8 and 1.0 mM BAK using the filtration method at 5 mg/mL DNA provided similar results. These results suggest that plasmid DNA binds to CRL1005 particles in the presence of BAK and that the amount of DNA binding at 0.6 mM BAK is ~100–150 μg/mL, which represents ~2–3% of the total DNA. It is not entirely clear why the results for the centrifugation and filtration methods differ significantly above 0.6 mM BAK but it seems likely that some of the bound DNA may be lost from the DNA-CRL1005-BAK particles during the centrifugation method because the sample gets diluted by ~16-fold during centrifugation. We do not believe that the presence of DNA-BAK precipitates was responsible for these differences, because there is no evidence for their existence above the cloud point of the formulation (Fig. 7, discussed below).

To determine how DNA binding to CRL1005 particles is affected by the DNA concentration, a sample containing 0.6 mM BAK and 7.5 mg/mL CRL1005 was titrated with increasing amounts of DNA and the amount of DNA bound to the CRL1005 particles was determined by filtration. The results shown in Figure 4B indicate that the amount of DNA bound to the CRL1005 particles saturated at ~135 μg/mL DNA. Because ~100% of the DNA was bound to CRL1005 particles at concentrations ≤100 μg/mL, these data suggest that DNA binds to CRL1005-BAK particles with relatively high affinity. The maximum capacity for binding of DNA in this formulation (~135 μg/mL) corresponds to ~2.7% of the total DNA in a formulation containing 5 mg/mL plasmid DNA.

Microscopic Visualization of Plasmid DNA Bound to CRL1005-BAK Particles

To determine whether DNA bound to CRL1005 particles could be visualized by light microscopy, we first formed CRL1005 particles of ~2-μm diameter by warming a cold solution of CRL1005 (7.5 mg/mL) in PBS to room temperature. BAK (0.6 mM) was then added to allow for binding to the preformed CRL1005 particles. After addition of the DNA-specific fluorescent label PicoGreen (Molecular Probes) and 0.3 mg/mL plasmid DNA, the particles were observed using a fluorescent microscope. The images shown in Figure 5 clearly

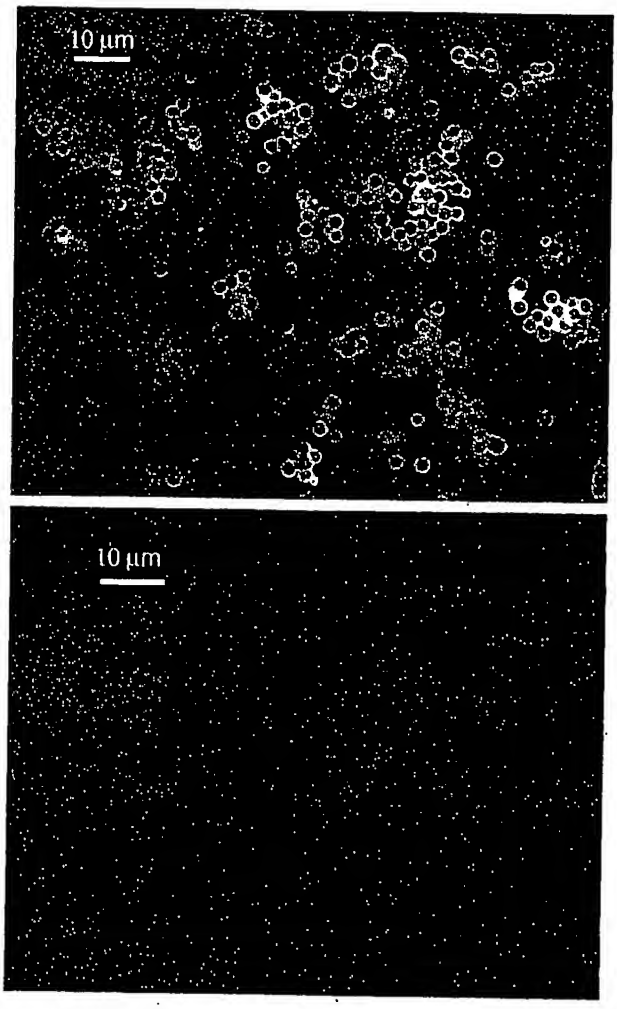

Figure 5. Fluorescence microscopy of CRL1005 particles in the presence of DNA. CRL1005 particles were prepared in the absence of BAK (~2-μm diameter). After particle formation, BAK, the DNA fluorescent probe PicoGreen, and DNA were added in order (upper panel). A DNA concentration of 0.3 mg/mL was used to reduce background fluorescence. As a control, a sample was prepared by the above procedure, but without BAK (lower panel).

show fluorescent rings of labeled DNA of the expected size. The results show no evidence of DNA binding to CRL1005 particles in the absence of BAK.

Although the composition of the formulation prepared for the visualization of DNA binding to CRL1005-BAK particles was not exactly the same as the formulation used in the rhesus monkey studies (D118), the same CRL1005 concentration (7.5 mg/mL) and buffer (PBS) were used. Because the particles in the D118 formulation were too small for effective visualization (~300 nm), it was necessary to generate ~2-micron particles by slowly warming the CRL1005 solution through the phase-transition temperature. However, the results in Figure 2 indicate that the CRL1005 particle size does not affect the binding of BAK; therefore, the amount of BAK bound to the CRL1005 particles in this formulation was the same as in D118. Because the use of 5 mg/mL plasmid DNA produced excessively high background fluorescence, the DNA concentration was reduced to 0.3 mg/mL. However, this change would not be expected to reduce the amount of DNA bound to the CRL1005-BAK particles, because the results shown in Figure 4B show that the binding of plasmid DNA to CRL1005-BAK particles saturates at ~150 μg/mL plasmid DNA. Therefore, these results strongly suggest that plasmid DNA binds to the surface of CRL1005-BAK particles in the same formulation used to generate the immunogenicity results in rhesus monkeys (D118).

Effect of Other Cationic Surfactants on the Binding of DNA to CRL1005 Particles

To determine whether other cationic surfactants would enhance the binding of plasmid DNA to CRL1005 particles, we titrated samples of 7.5 mg/mL CRL1005 with increasing concentrations of CPC and CTAC, then separated bound from free DNA. The results shown in Figure 6 show that each of these surfactants was able to support the binding of DNA to CRL1005 particles at concentrations above 0.2 mM, in a concentration-dependent manner. The results also show that the level of DNA binding was similar for each surfactant and similar to the results with BAK, shown in Figure 4A. Interestingly, these data also suggest the existence of a threshold of surfactant concentration required for the binding of DNA, but the threshold appears to be at ~0.1–0.2 mM CPC/CTAC. In a separate study, we titrated a formulation containing 22.5 mg/mL CRL1005 and 5 mg/mL DNA with increasing concentrations of CPC and CTAC and the results clearly showed a threshold at 0.5 mM for both surfactants (data not shown). If our hypothesis for the existence of this threshold is correct, we would expect the threshold to be approximately threefold higher at 22.5 mg/mL than at 7.5 mg/mL and this would correspond to a threshold of ~0.17 mM surfactant for CPC and CTAC with 7.5 mg/mL CRL1005 (a result that is consistent with the data shown in Fig. 6).

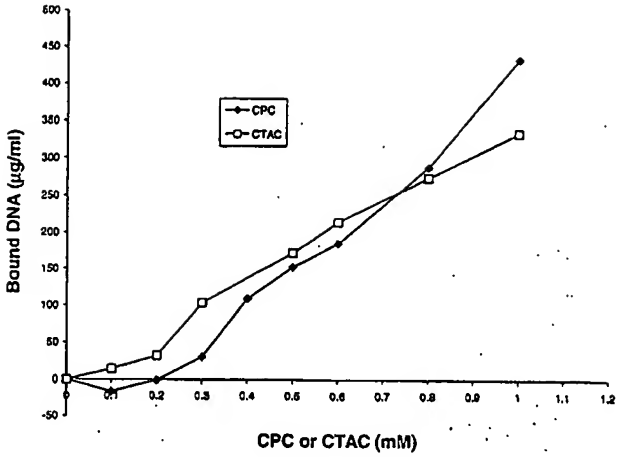

Figure 6. Binding of DNA to CRL1005 particles in the presence of CPC or CTAC. Samples contained 5 mg/mL plasmid DNA, 7.5 mg/mL CRL1005, and one of the surfactants at the indicated concentrations. Free DNA was separated from DNA bound to CRL1005 particles using the filtration method. The amount of DNA bound to CRL1005 particles is expressed as micrograms/milliliter in a sample containing 5 mg/mL plasmid DNA.

Dissolution of DNA-BAK Precipitates by CRL1005

It has been reported that DNA-BAK precipitates are able to enhance the immune response induced by plasmid DNA in mice.[15] Based on this report, it seemed possible that the immune response induced by DNA/CRL1005/BAK formulations (see Fig. 1) might have been due to the presence of DNA-BAK precipitates. To determine whether DNA-BAK precipitates are present in DNA/CRL1005/BAK formulations above the cloud point, PBS formulations containing DNA only, DNA-BAK, or DNA/CRL1005/BAK were subjected to a step centrifugation method to separate free DNA from DNA-BAK precipitates and from CRL1005-BAK-DNA particles. The results, shown in Figure 7, indicate that there was ~100 μg of residual DNA recovered from the bottom of the centrifuge tube in the absence of

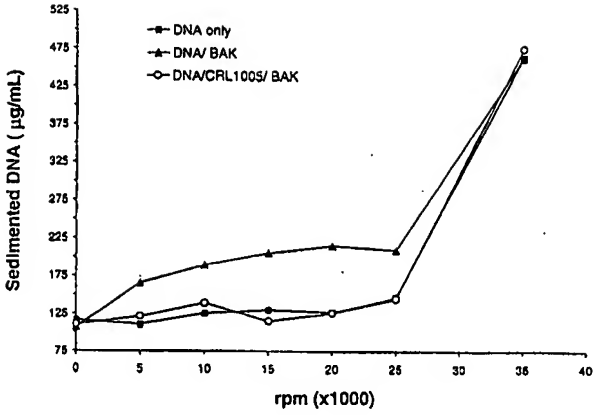

Figure 7. Complete dissolution of DNA/BAK precipitates above the cloud point of CRL1005. DNA sedimented during 30-min centrifugation steps at various speeds for solutions containing only plasmid DNA, or a solution containing 5 mg/mL DNA and 0.5 mM BAK, or a solution containing 5 mg/mL DNA + 7.5 mg/mL CRL1005 + 0.5 mM BAK. The amount of DNA sedimented is expressed as micrograms/milliliter in a sample containing 5 mg/mL plasmid DNA.

centrifugation for the DNA-only control; therefore, this level of DNA was considered background. The amount of DNA pelleted in the DNA-only control did not substantially increase above the background level until the centrifugation speed exceeded 25,000 rpm; therefore, no significant amount of free DNA was pelleted at or below 25,000 rpm. Moreover, there was no significant difference in the amount of DNA sedimented at ≤25,000 rpm between the DNA control and the DNA/CRL1005/BAK formulation, suggesting that DNA-BAK precipitates were not present (CRL1005-BAK-DNA particles did not sediment until >35,000 rpm). However, in formulations containing DNA and BAK (without CRL1005), the amount of sedimented DNA was significantly greater than that of the DNA control or the DNA/CRL1005/BAK formulation (~100 µg/mL over background at 20,000 rpm). Therefore, these data indicate that there was no detectable amount of DNA-BAK precipitate present in the DNA/CRL1005/BAK formulation above the cloud point. Because visual observation of this formulation below the cloud point (data not shown) clearly reveals the presence of DNA-BAK precipitates (in contrast, CRL1005/DNA formulations are completely clear below the cloud point), these results suggest that CRL1005 particles completely dissolve any DNA-BAK particulates. This conclusion is also supported by the light-scattering measurements of D118, shown in Figure 1B, which show no indication of the presence of DNA-BAK precipitates above the cloud point. Additional light-scattering measurements of D118-like formulations containing even higher concentrations of BAK (up to 1.0 mM) also showed no evidence for the presence of DNA-BAK precipitates (data not shown). Taken together, these data indicate that the enhancement of cellular immunity induced by the D118 formulation was not due to DNA-BAK precipitates.

DISCUSSION

Based on the need to identify safe and effective adjuvants for plasmid DNA vaccines, we began this work by exploring the adjuvant properties of CRL1005. The initial animal studies showed that CRL1005 enhances the cellular immune response induced by HIV-1 *gag* plasmid DNA in rhesus monkeys. Because the adjuvant properties of CRL1005 are believed to be related to its ability to form particles,[6,7] we conducted biophysical characterization studies in an attempt to correlate the physical/chemical properties of the formulation with the enhanced immune response. These initial studies included measurements of associated DNA. We thought it unlikely that the CRL1005 particles would bind plasmid DNA, because it is a nonionic polymer, and our results confirmed this hypothesis. However, we believed that a hydrophobic cationic surfactant would be likely to bind to the surface of the CRL1005 particles rendering them capable of binding DNA through electrostatic interactions. Based on previous reports of enhanced DNA delivery by cationic microparticles with DNA adsorbed to the surface,[16,17] we hypothesized that cationic microparticles of CRL1005, produced by formulating CRL1005 with a cationic surfactant, might also enhance DNA delivery. We selected BAK as the cationic surfactant to test this hypothesis, in rhesus immunogenicity studies, based on its history of use in pharmaceutical products.[18] Moreover, the BAK concentration was selected to be within the concentration range used in previous pharmaceuticals, to minimize the possibility of adverse responses. The DNA concentration was selected on the basis of previous immunogenicity results using nonadjuvanted DNA formulations.

The immunogenicity and characterization results demonstrate that the addition of BAK to a

DNA/CRL1005 formulation leads to the formation of CRL1005-BAK-DNA particles and to an enhancement of the cellular immune response in rhesus monkeys. The mechanism for how the CRL1005-based plasmid DNA formulations enhance the immune response is unknown; however, it seems possible that the DNA-CRL1005-BAK particles may be acting as an adjuvant and/or enhancing the delivery of plasmid DNA.

Aluminum phosphate is a negatively charged particle-based adjuvant that is capable of enhancing the immune response induced by DNA vaccines.[6,19–21] Although aluminum phosphate particles are much larger than the DNA-CRL1005-BAK particles, the particulate nature and negative surface charge of the DNA-CRL1005-BAK particles suggest that they may be functioning similarly to aluminum phosphate. However, there is also significant support for the hypothesis that cationic microparticles can enhance the delivery of DNA, *in vivo*. One such cationic microparticle formulation is based on the use of PLG (polylactide-*co*-glycolide) and CTAB (cetyltrimethylammonium bromide) to produce cationic microparticles that bind DNA.[16] The PLG-CTAB microparticles are reported to be more effective than naked DNA for induction of immune responses in mice, guinea pigs, and rhesus monkeys.[22] Moreover, the mechanism of action appears to be, at least in part, the facilitation of DNA uptake by antigen-presenting cells.[23] Another type of cationic microparticle formulation being evaluated as a gene-delivery vehicle was prepared by adding the cationic lipid DOTAP (1,2-dioleoyl-3-trimethylammonium-propane) to an oil-in-water emulsion (MF59 and other emulsions) to make a cationic submicron emulsion with a particle size near 180 nm. Based on immunogenicity data generated in mice and rabbits, the MF59/DOTAP emulsion appears to be an effective delivery system for plasmid DNA.[17]

A significant difference between the previously described cationic microparticle formulations and CRL1005-based formulations containing BAK, is the polarity of the surface charge after DNA binding. For the two cationic microparticle formulations described above, the net surface charge after binding DNA is positive,[16,17] but CRL1005-BAK-DNA particles have a negative surface charge. The significance of surface-charge polarity on the type and/or magnitude of the immune responses induced by plasmid DNA is unknown, but the difference suggests that CRL1005-BAK-DNA particles may not have the same mechanism of action as the cationic microparticle formulations previously described. It should also be noted that Prokop et al.[24] evaluated a series of cationic polymers for their ability to enhance *in vivo* gene delivery and showed that the most effective polymers were members of the Tetronic polymer series (polyethylene–polypropylene block copolymers; BASF) that formed negatively charged particles with DNA. Therefore, the influence of surface-charge polarity on the ability of a microparticle carrier to deliver plasmid DNA may be different in different formulations or in different animal species.

One characteristic of the DNA/CRL1005/BAK formulation suggesting that increased DNA delivery is an unlikely explanation for the enhanced immune response is the low level of DNA associated to the CRL1005-BAK particles. In the D118 formulation containing 0.5–0.6 mM BAK, the amount of DNA associated to CRL1005-BAK particles is ~1.5–3% of the total DNA (at 5 mg/mL). Although the level of bound DNA is a small fraction of the total, it corresponds (by calculation) to ~20–40 molecules of plasmid bound to each of the $\sim 5 \times 10^{11}$ CRL1005 particles/mL (based on a monodisperse population of ~300-nm spherical particles and a 7-Kb plasmid). Based on the high concentration of DNA-CRL1005-BAK particles and the presence of multiple copies of plasmid on each particle, these calculations suggest that enhanced DNA delivery is a reasonable hypothesis to explain the increased immune response caused by the addition of BAK to the DNA/CRL1005 formulation. Unfortunately, it is not possible to address this hypothesis in animals by testing a formulation containing only ~150 μg/mL total DNA, to eliminate the immune response induced by free DNA, because the use of DNA concentrations in this range results in extensive aggregation. Moreover, experiments to examine the delivery of plasmid by measuring transgene expression in mice does not appear to be feasible because of saturation of DNA delivery at DNA concentrations far below the 5 mg/mL DNA concentration in the D118 formulation. These results indicate that studies to address the mechanism of immune enhancement will need to be done in animals larger than mice and with formulations having a DNA concentration ≥~1 mg/mL.

Our results indicate that BAK acts to stabilize small (~300 nm) CRL1005 particles and to bridge the association of DNA to the particle surface. Moreover, based on the data in Table 3 and Figure 2, the BAK-14 and BAK-16 homologs were

nearly completely bound to CRL1005 particles, but ~75–80% of the BAK-12 remained unassociated. The effects of free BAK-12 on the immunogenicity are unknown, but the mere presence of free surfactant (~0.25 mM BAK-12 in D118 with 0.5 mM total BAK) in the formulation raises the possibility that it may facilitate DNA delivery by enhancing the permeability of cell membranes or by acting through an unknown pathway. However, the surfactant properties of BAK may also have an inhibitory effect on antigen-presenting cells. The results in Table 3 suggest that a CRL1005/BAK/DNA formulation containing only BAK-14 and/or BAK-16 (or CPC or CTAC) would have greatly reduced levels of free surfactant as well as a lower total surfactant concentration. Therefore, the effect of BAK on the immune response could be evaluated by conducting immunogenicity experiments comparing formulations prepared with the BAK mixture with those containing only BAK-14 or BAK-16.

Plasmid DNA and BAK surfactants form precipitates that have been reported to enhance DNA delivery.[15] Based on this report, an unexpected result from our studies is that DNA-BAK precipitates do not coexist with CRL1005-BAK-DNA particles (ternary complexes) in these formulations above the cloud point of CRL1005. However, DNA-BAK precipitates were observed in the D118 formulation below the cloud point (in the absence of CRL1005 particles). Further investigations using other biophysical techniques revealed a critical BAK concentration threshold required for interaction with DNA and that the free BAK concentration in the D118 formulation (above the cloud point) is below this threshold (results prepared for a separate publication). Taken together, these results suggest that BAK binds with higher affinity to CRL1005 particles than to plasmid DNA and that the presence of CRL1005 particles reduces the free BAK concentration below the threshold for interacting with DNA.

The DNA/CRL1005/BAK formulations described in this report appear to have significant advantages over other types of microparticle formulations in terms of the ease of manufacture and use, flexibility, and storage stability. They are liquid formulations, rather than lyophilized, and are therefore less costly to manufacture and easier to use because they do not require a separate diluent and a reconstitution step. Preparation of sterile vaccine formulations requires only the addition of sterile BAK to a solution of DNA/CRL1005 that is sterile-filtered below the cloud point. Moreover, the particle formation process for CRL1005 appears to be very reproducible. The size of CRL1005-BAK-DNA particles in the D118 formulation was consistently in the 250- to 350-nm range, even after repeated freeze/thaw/warming cycles, and was not significantly affected by the warming rate through the cloud point (data not shown).

In terms of flexibility, we have found that the formulation composition, surfactant type and concentration, and the CRL1005 concentration, can be adjusted to produce particle sizes from ~150 nm to 2–3 microns and DNA loading levels from 0 to >10,000 copies of plasmid per CRL1005 particle (unpublished data). Although CRL1005 particles appear to bind surfactants through hydrophobic interactions, the zeta potential measurements indicate that a fraction of the hydrophilic head groups are located on the surface of the particle and can bind to DNA molecules in solution. These data suggest that systematically altering the chemical structure of the surfactant headgroup might provide a convenient approach for exploring the effects of surface chemistry on the ability of CRL1005 particles to act as an adjuvant or a carrier for plasmid DNA vaccines.

Safety of the excipients used in a parenteral formulation is an important factor affecting regulatory approval and the acceptability of a vaccine. Therefore, it is highly desirable to use excipients already approved by regulatory agencies for new vaccine formulations. With regard to DNA/CRL1005/BAK formulations, BAK is approved by the FDA for use as a preservative.[25] Although it is most often used in ophthalmic products,[18] it is also approved for im injection.[25] CRL1005 has been evaluated in a Phase I clinical trial at doses up to 75 mg im without evidence of significant local or systemic toxicity.[26] Moreover, DNA/CRL1005/BAK formulations were well tolerated by both mice and rhesus monkeys (data not shown). These data suggest that the DNA/CRL1005/BAK formulation (D118) would be reasonable to test for use in humans.

The usefulness of a vaccine formulation can be limited by insufficient storage stability. Therefore, one of the goals of our laboratory is the development of DNA/CRL1005/BAK formulations that are stable for at least 2 years, when stored at 2–8°C. Compared with most protein-based or live-virus-based vaccines, plasmid DNA is very stable. In fact, we have previously reported the identification of plasmid DNA formulations (without BAK or CRL1005) that are stable for at least 2 years when

stored at room temperature.[27] However, the effects of BAK and CRL1005 on the stability of plasmid DNA have not been reported. In addition to any effect of CRL1005 and BAK on the stability of DNA, the stability of the CRL1005 polymer and BAK must also be ensured. BAK is known to be stable through autoclaving and is therefore unlikely to be a cause for concern.[18] However, POE-POP-POP polymers are known to degrade by autoxidation.[28] Therefore, the development of stable DNA/CRL1005/BAK formulations may require control of CRL1005 degradation. By monitoring both the physical and chemical stability of candidate formulations during storage, we have identified and characterized liquid formulations of DNA/CRL1005/BAK that are stable for at least 1 year at 2–8°C, suggesting that development of a stable liquid formulation is possible (results to be reported separately).

ACKNOWLEDGMENTS

The authors thank Robert Druilhet at the New Iberia Research Center for help with bleeding of and preparation of cells from rhesus monkeys, and P. K. Tsai and Joyce Sweeney for their help and advice on the development of analytical methods.

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JOURNAL OF Pharmaceutical Sciences

VOLUME 93 · NUMBER 7 · JULY 2004
hed Online in Wiley InterScience
pril 2004 - 13 May 2004

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Published in Cooperation with the American Association of Pharmaceutical Scientists

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